Prospective cytometric study evaluating the alkaline phosphatase activity as a potential functional biomarker of primitive malignant cells in acute leukemia

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INTRODUCTION
Alkaline phosphatase (ALP) is an enzyme highly expressed in primitive stem cells (SCs), including pluripotent SCs, embryonic SCs, and embryonic germ cells. ALP activity is altered in some disease conditions, such as leukemia. We previously adapted a fluorescence microscopy based method to detect ALP+ cells, and this new flow cytometric strategy was used to study the ALP activity in human leukemia in combination with immunophenotyping (LG Rico, 2016). The aim of this study was to use this rapid quantitative assay to study clinical relevance for early detection of ALP activity in a prospective monitoring of leukemic patients at diagnosis, post-treatment follow-up, and relapses to show that changes in the ALP levels can be used to detect rare populations of highly refractory malignant cells that can be involved in disease persistence and relapses.

MATERIALS AND METHODS
A total of 25 patients diagnosed with acute myeloid leukemia (AML) were included in this study. Peripheral blood and/or marrow specimens were analyzed prospectively at diagnosis, follow-up, and/or relapses. ALP staining was combined with CD34, CD38, CD90, CD117 and CD123 immunophenotyping and no-lyse no-wash methods using the Attune™ Nxt Flow Cytometer (Thermo Fisher). Vybrant™ DyeCycle™ Violet Stain (DCV) was used to discriminate nucleated cells from erythrocytes and debris. Alkaline Phosphatase Live Stain (APLS) was obtained from Thermo Fisher. Clinical data from cytology morphology analysis, immunophenotyping, karyotype and molecular biology was also used in this investigation. APLS was used as described previously (LG Rico, 2016). Statistical analysis was performed using RStudio. Two-sided Fisher’s exact tests were used to test for differences between categorical variables. Two-sided Wilcoxon rank sum tests were used to compare continuous variables.

RESULTS AND DISCUSSION I
We implemented a novel multicolor panel for the screening of ALP+ cells in leukemia, consisting of 7-colors and 4-laser excitation. Figure 1 shows a representative acquisition protocol with the gating strategy and representative dot plots to analyze the ALP activity in combination with immunophenotyping. The no-lyse no-wash strategy allows us to preserve the ALP function and detect rare events more accurately, especially in samples from patients in treatment. The use of 4-laser excitation allowed us to minimize the color compensation.

RESULTS AND DISCUSSION II
ALP activity was studied in 25 AML patients at diagnosis (from a total of 34) and were classified in two groups according to the numbers of leukemic blasts expressing ≥10% of ALP+ cells and less than 10% of ALP+ cells. Differences between the two groups were analyzed. Table 1 shows AML patient characteristics between APLS≥10% (n=17) and APLS<10% (n=8) groups. From those patients with more than 10% of ALP+ leukemic cells at diagnosis, 16 relapsed (94.1%), showing significant differences when compared to the APLS<10% group (P=0.001; 95% CI = 0.00-0.36), giving support to the hypothesis that ALP activity at diagnosis may predict relapses and disease persistence. Four patients within the APLS≥10% group had CD34–/CD123+/CD117+ leukemic cells with significant differences (P=0.023; 95% CI = 1.02-2.89) when compared with the group of more than 10% of ALP positive cells, indicating that phenotype may be associated with better outcome than the CD34+ phenotype. No differences were found when comparing risk cytogenetic classification between both groups.

CONCLUSIONS
Our results suggest that ALP+ leukemic cells appear to sustain leukemogenesis over time and may be related to relapses and therapy resistance. This new cytometric functional approach may help to better identify leukemic cells that remain elusive using current optimized multicolor panels for leukemia immunophenotyping. Increased numbers of primitive ALP+ leukemic cells appear to be a good predictor of relapse and treatment refractoriness. Further experiments will be needed, such as cell sorting, serial transplantation assays and genome-wide transcriptome studies to enlighten the role of primitive subsets of ALP+ cells in blood cancer.

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